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Secondary Immune Surveillance via upregulation of C-type lectin receptors

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C-type lectin receptors (CLRs) are a family of pattern recognition receptors that sense diverse microbial and damage-associated molecular patterns. Many CLRs, like Mincle and Dectin-3, are upregulated by TLR4 signaling. In Alcohol-associated Hepatitis (AH), where alcohol consumption leads to leakage of bacterial LPS, TLR4 upregulates CLR expression and increases the diversity of what the immune system can detect, which we call "secondary immune surveillance". Previously, we found human monocytes upregulate CLRs in response to LPS/TLR4 signaling with much higher expression in AH. In this study, we broaden our understanding of secondary immune surveillance. We find that CLRs are upregulated by many kinds of TLR signaling. Additionally, mouse monocytes and microglia also upregulated CLRs in response to LPS/TLR4 signaling. We used Mincle-KO mice to dissect how loss of one CLR impacts tissue inflammation. Mincle-KO mice were protected from liver inflammation caused by LPS, but experienced worse adipose tissue inflammation, suggesting a shift in the site of injury. Bulk RNA-seq revealed extensive alternative splicing of many CLR genes, with only translatable mRNA detected after LPS. Finally, using bulk RNA-seq data of livers from patients with AH, we observe extensive expression of CLRs, suggesting these receptors may betargets for therapies to reduce inflammation.

Chronic liver diseases are associated with dysfunctional metabolic pathways, reduced regeneration, and increased infiltration of immune cells into the liver parenchyma[1,2]. Alcohol-associated Liver Disease (ALD) is a spectrum of disorders that alter the metabolic, regenerative, and innate immune functions of the liver. In extreme cases, like Alcohol-associated Hepatitis (AH), infiltrating immune cells dramatically increase inflammation in the liver, leading to further damage to hepatocytes[1–3]. AH has the highest 30-day mortality rate of all chronic liver diseases with the number of patients increasing in recent years[4], so we have to fully understand what drives immune cell infiltration in this disease.

Pattern recognition receptors (PRRs) are important factors for the inflammatory response in the liver and development of ALD and AH in a myriad of ways. For example, TLR4 senses bacterial lipopolysaccharides that leak from the gut during alcohol consumption[5], and inhibition of TLR4 can reduce liver inflammation and damage[6]. Mincle is a C-type Lectin Receptor (CLR) in the family of PRRs that senses diverse Pathogen and Damage-associated Molecular Patterns (PAMPs and DAMPs)[7,8]. Mincle, which is primarily expressed by monocytes and macrophages, senses SAP130, a DAMP secreted by dying hepatocytes, and drives liver infiltration in response to damage[7,9,10]. Mincle-KO mice are protected from ALD due to reduced infiltration of immune cells into the liver, suggesting infiltrating monocytes play an important role in disease progression[9]. Mincle signals through SYK signaling, and inhibition of SYK can also ameliorate ALD, suggesting that infiltrating monocytes alone do not lead to liver damage but rather downstream pro-inflammatory gene expression causes ALD[11].

Mincle is upregulated by LPS/TLR4 through NF-κB signaling[9]. In the genome, Mincle (Clec4e) is in a region called the NK-gene-receptor complex, which houses a large number of CLR genes important for NK-cell, monocyte, and dendritic cell function[12–15]. This region is mostly very well conserved between mice (chromosome 6)[16], rats (chromosome 4)[17], and humans (chromosome 12)[18], although there are notable differences. Our group previously showed that Mincle and other neighboring CLRs, like Dectin-3, Dectin-2, and DCIR, are upregulated by LPS/TLR4 signaling in human peripheral blood mononuclear cells (PBMCs) and have exacerbated expression in AH[19]. Using single-cell RNA sequencing (scRNA-seq), we found that neighboring genes, specifically Dectin-3, Mincle, and a nearby long non-coding RNA, had highly co-regulated expression in response to LPS, and in AH, neighboring DCIR was also highly correlated[19]. Using high-throughput chromatin conformation capture technology (Hi-C), we found that Mincle, Dectin-3, and Dectin-2 were within the same topologically-associated domain, suggesting a possible mechanism for the co-regulation of these genes[20].

CLRs are PRRs that sense a large diversity of fungal, bacterial, and parasitic PAMPs[8], and distinct tissue specific DAMPs[21]. Upregulation of

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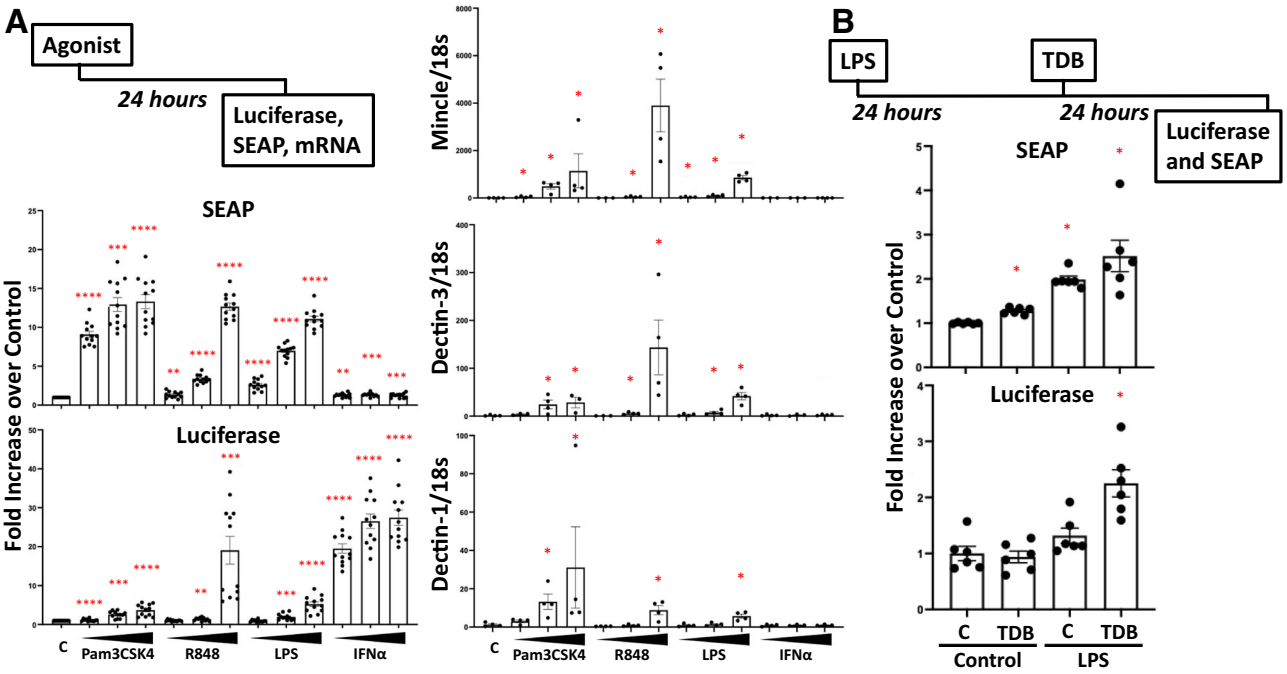

Fig. 1 | THP1-Dual cells were challenged with different innate immune agonists then supernatants were collected to measure SEAP (NF-κB signaling) and Luciferase (Interferon signaling). RNA was also isolated from cells for qPCR measurements of gene expression. **A** Cells were challenged with Pam3CSK4 (10 ng/ml, 1 μg/ml, 10ug/ml), R848 (10 ng/ml, 1 μg/ml, 10ug/ml), LPS (100 pg/ml, 1 ng/ml, 10 ng/ml), and IFNα (10 ng/ml, 50 ng/ml, 100 ng/ml) for 24 hours, then supernatants were collected for SEAP and Luciferase measurement and RNA isolated from cells for qPCR. **B** Cells were challenged with LPS (100 pg/ml) for 24 h, then TDB (10 μg/ml) for 24 h, then supernatants were collected for SEAP and Luciferase measurements. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparisons test ($p < 0.05$).

CLRs enables the immune system to detect more deleterious pathogens and damaged tissues, which we have termed this process as "secondary immune surveillance"[19]. Secondary immune surveillance is an important aspect of innate immunity, where potent immune agonists like LPS/TLR4 signaling can act as a primary signal and then upregulate CLRs which detect less abundant PAMPs and DAMPs and sense tissue damage that might be concurrent. In ALD, we hypothesize that secondary immune surveillance, via CLRs, is essential for detecting other pathogens that leak from the gut beyond just LPS containing bacteria.

While Mincle inhibition reduces liver inflammation, Mincle also contributes to inflammation in other tissues like gonadal adipose tissue, though the mechanism is less well understood[22]. Unfortunately, very little is known about these neighboring CLRs like Dectin-3, Dectin-2, and DCIR in liver and adipose tissue inflammation. Mincle-KO mice have shown some reduction in adipose tissue inflammation in mouse models of obesity[22,23]. In these studies, Mincle-KO mice are not protected from obesity as weight gain and adipose tissue accumulation were not reduced after 16 weeks of high-fat diet. In the adipose tissue, Mincle-KO mice did not reduce infiltrating immune cells, but the number of crown-like structures and interstitial fibrosis was decreased, suggesting Mincle is important in the adipose tissue microenvironment and clearance of dead cells, but may not play a role in overall obesity. Importantly though, these mice were protected from liver steatosis, suggesting Mincle has a more important role in liver disease progression than adipose tissue.

In this study, our goal is to broaden our understanding of secondary immune surveillance in both mice and humans and across different tissues. We start by expanding what upregulates these CLR genes in the NK-gene receptor complex. Then using a common model of LPS injection as a simple way to upregulate these genes in mice, we characterize diverse CLR expression patterns in myeloid cells in different tissues. Then we describe how LPS injection affects the Mincle-KO mice, where Mincle cannot be upregulated by LPS, but all other CLRs are upregulated, and the impact this has on tissue inflammation, specifically the liver and adipose. Ultimately, we sought to better describe the conditions for secondary immune surveillance, and the impact it has on tissue inflammation.

Results

Mincle and Dectin-3 are upregulated by NF-KB signaling

Mincle and other CLRs in the NK gene receptor complex are upregulated by LPS (TLR4) signaling[9,19] so we hypothesize that all TLR signaling, via NF-κB, will upregulate these genes. THP1-Dual cells are a modified version of the THP1 monocytic cell line where two reporter constructs have been added to the genome: a secreted alkaline phosphatase (SEAP) gene downstream of an NF-κB promoter and secreted luciferase downstream of the interferon (IRF) promoter. Using these cells, we can monitor the dual activation of NF-κB and interferon signaling and by qPCR, we can measure CLR expression. We challenged these cells with a variety of TLR agonists and Interferon α and measured SEAP and Luciferase in cell culture supernatants and Mincle, Dectin-3, and Dectin-1 by qPCR. Most of these agonists target plasma membrane localized TLRs and preferentially signal through NF-κB, including Pam3CSK4/TLR2, LPS/TLR4, and R848/TLR7 (Fig. 1A). While Pam3CSK4/TLR2 and LPS/TLR4 could dually upregulate both pathways, there was a clear bias toward NF-κB activation. R848/TLR7 highly upregulated both the NF-κB and interferon pathways. Interferon α, as expected, only upregulated luciferase secretion. Mincle, Dectin-3, and Dectin-1 were highly upregulated by all TLR agonists in a dose dependent manner suggesting secondary immune surveillance via expression of diverse CLRs is a feature of NF-κB signaling. Interferon α did not upregulate CLR expression.

TLR signaling can upregulate both NF-κB and interferon pathways, with a bias toward NF-κB. Next, we wanted to know if Mincle, via Syk signaling, also upregulates both pathways. To test this, we challenged THP1-Dual cells with LPS (100 pg/ml) for 24 h to induce Mincle expression, but at

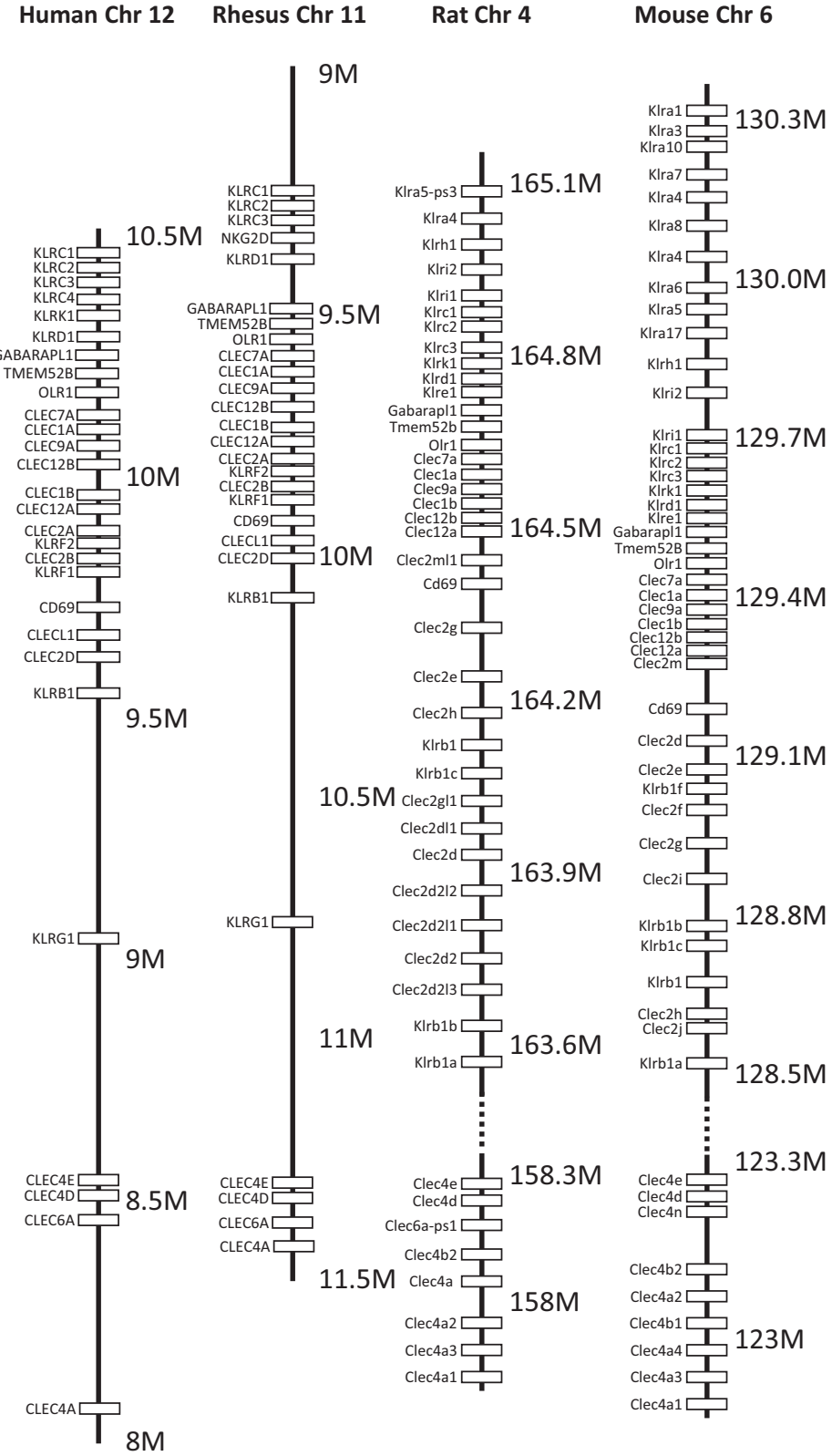

Fig. 2 | Schematic comparing CLRs in the NK-gene receptor complex on different species.

this low concentration, SEAP is minimally induced while Luciferase is not expressed at all. These cells were then challenged with the Mincle agonist Trehalose-6,6-dibehenate (TDB), which is a synthetic analog of cord factor, both of which are potent Mincle agonist[24]. Interestingly, both SEAP and Luciferase were induced by LPS + TDB, suggesting Mincle, via Syk signaling, can activate both pathways (Fig. 1B).

Highly diverse monocytes have distinct CLR expression patterns

The NK-gene receptor complex, which houses many CLR genes, is highly conserved between mouse and human, but there are large structural differences mostly due to an expansion of many genes in rodents like rats and mice[15] (Fig. 2). Specifically, DCIR (Clec4a) is expanded to 6 genes in mice (Clec4a1, Clec4a2, Clec4a3, Clec4a4, Clec4b1, and Clec4b2) and the Clec2

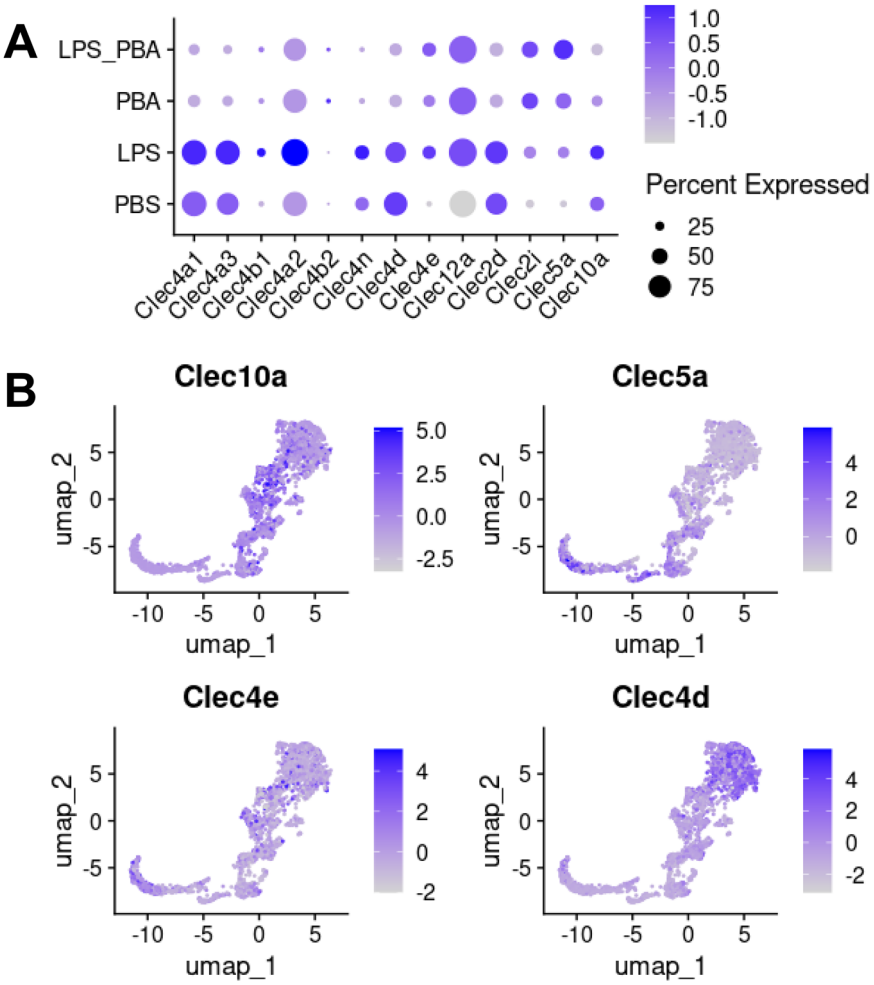

Fig. 3 | scRNA-seq data from mouse monocytes cultured from bone marrow after low-dose LPS (100 pg/ml) or 4-phenylbutyrate (PBA,1 mM) challenge for 5 days [GEO: GSE160450][26]. **A** Dot plot showing expression of CLRs in all monocytes. **B** UMAP plots showing expression pattern of CLRs in monocytes.

and KLR genes in humans are also significantly expanded and more complex in mice.

While LPS (TLR4) signaling has been shown to upregulate Mincle in mice[9], little is known about the regulation of the other CLRs in mouse monocytes. We utilized a dataset where monocytes were cultured from mouse bone marrow, then challenged with low-dose LPS (100 pg/mL) for 5 days. In contrast, monocytes were also challenged with 4-phenylbutyrate (PBA,1 mM) for its role in suppressing LPS signaling[25]. Monocytes were then collected and analyzed by scRNA-seq [GEO: GSE160450][26]. From this dataset, we measured changes in CLR expression in response to LPS or PBA. Without any induction, monocytes expressed varying levels of Clec4a1, Clec4a3, Clec4b2, Clec4d, and Clec2d (Fig. 3A). In response to LPS, almost all of these CLRs had increased expression both in terms of percentage of cells and expression level, including Mincle (Clec4e). As expected, PBA did not significantly induce CLR expression and in combination, PBA suppressed LPS-induced upregulation of CLRs. Monocytes are highly diverse cells, and the expression of these CLRs seems to vary throughout the population (Fig. 3B).

Mincle upregulation by peripheral monocytes plays an important role in infiltration into the liver during ALD[9]. If many CLRs are upregulated by LPS/TLR4 signaling, we wanted to know how these molecules might affect inflammation in other tissues. We utilized a dataset where mice were given LPS by IP injection (10 mg/kg), then microglia were isolated from the brain and subjected to bulk-RNAseq [GEO: GSE75246][27]. In microglia, many CLRs were upregulated in response to LPS, including Mincle. Interestingly, Mincle was not the predominant CLR expressed, but rather Clec4a1, Clec4a3, and Clec4a2 were drastically upregulated by LPS (Fig. 4). These results show that other myeloid cells beyond monocytes upregulate CLRs in response to LPS/TLR4 and there is heterogeneity in CLR gene expression among these highly diverse cells and cell types.

Mincle-KO mice are protected from acute Liver Inflammation

Mincle-KO mice have been shown to be protected from mouse models of ALD due to reduced infiltrating pro-inflammatory monocytes[9,11]. ALD is complex because alcohol metabolism damages liver hepatocytes and causes gut barrier dysfunction leading to LPS and other gut contents leaking into portal circulation[1,28]. Both LPS/TLR4 signaling and hepatocyte damage are required for monocyte infiltration by Mincle. To simplify this pathway, we challenged Mincle knockout mice (Mincle-KO), heterozygous Mincle mice (Mincle-Het), and WT littermates with a low-dose IP injection of LPS (0.7 mg/kg) for 24 h to simulate low-levels of endotoxin exposure during alcohol consumption[29]. At this concentration, liver damage is minimal, as measured by ALT and AST (Supplementary Fig. 1) and hematoxylin and eosin staining (Supplementary Figs. 2 and 3). While this model causes minimal liver damage, LPS still induces liver inflammation, as measured by TNF expression in WT male mice, while Mincle-KO mice are protected from inflammation (Fig. 5). Mincle-Het mice, like WT, had similar levels of inflammation caused by LPS. Similarly, we measured Ly6C and Ly6G as markers of infiltrating monocytes and neutrophils, respectively, and observed increased expression in response to LPS in WT mice, while Mincle-KO had reduced infiltration. Mincle-Het mice had an intermediate phenotype compared to WT and Mincle-KO. Interestingly, female mice of all genotypes had very little liver inflammation or markers of infiltrating cells in response to LPS.

If Mincle-KO mice are protected from LPS-induced liver inflammation, we hypothesized that there might be negative consequences for

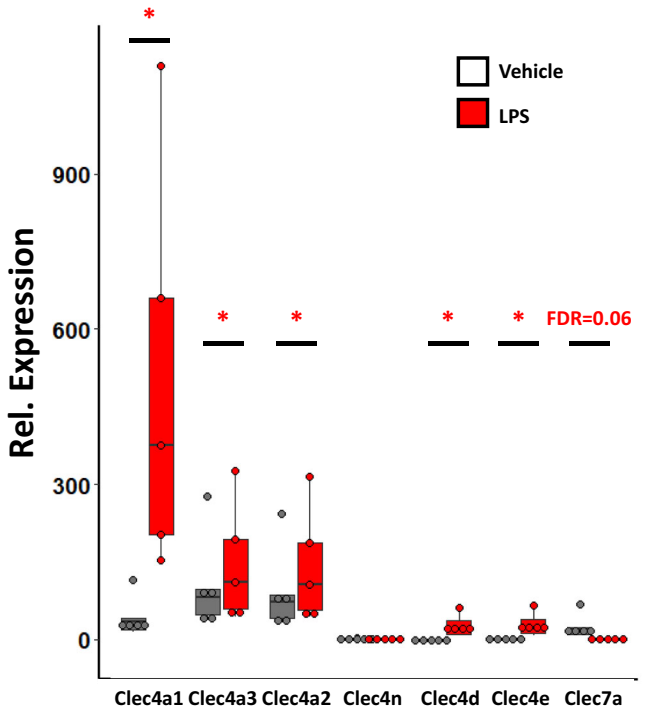

Fig. 4 | Bulk RNA-seq of microglia isolated from mice after LPS administration by IP injection (10 mg/kg) [GEO: GSE75246][27]. *Indicates $q < 0.05$ compared to control.

other tissues suggesting we are moving the inflammation from one site to a different site. Previous work found that while Mincle is expressed in adipose crown-like structures during high-fat diet, Mincle-KO mice are not protected from obesity or adipose tissue inflammation[22,23]. In response to low-dose LPS, adipose tissue inflammation varied considerably between males and females and all genotypes. In general, female WT and Mincle-Hets did not experience LPS-induced adipose tissue inflammation, but Mincle-KO mice had exacerbated adipose tissue inflammation, suggesting a movement of the inflammation from the liver to the adipose tissue in the absence of Mincle (Fig. 5). On the other hand, almost all of the male mice, regardless of Mincle genotype, experienced adipose tissue inflammation in response to LPS, suggesting immune cell infiltration in adipose tissue does not depend on Mincle expression. Similarly, hematoxylin and eosin staining of the adipose tissue revealed extensive infiltrating immune cells in response to LPS in male mice, but not female (Supplementary Figs. 4 and 5). These data suggest that acute LPS challenge caused worse adipose tissue inflammation in male mice than female mice, regardless of Mincle expression, and Mincle is not required for immune cell infiltration in adipose.

CLRs are alternatively spliced in response to LPS

We next performed bulk RNA-seq on the livers from these mice to investigate changes in CLR expression due to LPS in the different Mincle genetic backgrounds (WT, Het, and KO) (Supplementary Table 1 and 2). Unexpectedly, in response to LPS, we did not observe a strong induction of Mincle in the livers of the WT mice, which might be due to the relatively low concentration of LPS or Mincle drives infiltration only during chronic liver injury. Using the RNA-seq data, we analyzed changes in Mincle splicing and found that most of the Mincle transcripts in the liver were an isoform with a retained intron (between exons 4 and 5), which would lead to a non-protein coding transcript (Fig. 6). LPS-induced expression of other CLRs, like Dectin-3, Dectin-2, Clec4a1 and Clec4a3 (Fig. 6, Supplementary Fig. 6). Both Dectin-2 and Dectin-3 have alternative splice isoforms and LPS increased expression of the correct isoform that codes functional proteins (Fig. 6).

To better understand gene expression differences between WT, Mincle-Het and Mincle-KO mice, we performed weighted gene correlation network analysis (WGCNA). WGCNA determines how groups of genes with highly correlated expression are affected by external factors, which for this experiment would predominantly be due to Mincle expression level, Mincle genotype (WT, Het, KO), or LPS. As expected, WGCNA revealed large groups of genes (modules) with highly correlated expression (Supplementary Fig. 7). One of these modules (Brown) was highly correlated with LPS challenge and includes many genes associated with an immune response including both pro-inflammatory genes like Tnf and interferon associated genes like Ifit1. The Brown module also contained genes associated with Kupffer cells and reparative macrophages like Clec4f and Marco.

Mincle gene expression was most well correlated with the Pink module while Mincle genotype (WT, Het, KO), was most closely associated with the Tan module, though the Pink and Tan modules along with Magenta are adjacent and share many features (Supplementary Tables 3 and 4). These modules were also associated with many of the other CLRs, including Clec4a2, Clec2e and Clec2d, suggesting common expression among activated myeloid cells (Supplementary Table 5).

NK gene receptor complex is dysregulated in liver disease

We previously found that Mincle and other CLRs are highly upregulated in PBMCs isolated from AH patients and expression is exacerbated after LPS challenge. To see if CLRs are upregulated in AH livers, we used bulk RNA-seq data from patients with a variety of liver disease and measured gene expression throughout the region[30]. This dataset includes patients with early AH (EAH, $n = 12$), severe AH with liver failure (AHL, $n = 18$), explant tissue from patients with severe AH with emergency liver transplants (ExAH, $n = 10$), non-alcoholic fatty liver disease (NAFLD, $n = 8$), Hepatitis C virus (HCV, $n = 9$), and HCV with cirrhosis (HCV_Cirr, $n = 10$)[30]. Almost all CLRs and Killer-type Lectins in the region were differentially expressed in AH, with a few notable exceptions that were significantly downregulated (Fig. 7). Many of these CLRs, like DCIR, Dectin-3, Mincle, and Dectin-1, were significantly upregulated in the more severe AH patients, suggesting correlation between AH severity and CLR expression. Interestingly, CLEC5a, or MDL, which in a CLR on a different chromosome, was also upregulated in AH livers. These data suggest these genes contribute significantly to AH. Because these genes are predominantly expressed on myeloid cells like monocytes, macrophages, and neutrophils, as well as NK cells, these gene expression studies suggest an influx of these innate immune cells in the liver.

Discussion

The goal of this study was to broaden our understanding of how Mincle and CLRs in the NK gene receptor complex are regulated and co-regulated by innate immune signaling, their expression in different tissues, and impact they have during inflammation and disease progression. CLRs detect a large diversity of PAMPs that span all microbial communities from bacteria[31], viruses[32], fungi[33,34], and other eukaryotic parasites[15,35,36]. CLRs also detect a number of tissue-specific DAMPs making them equally important in responding to potentially deleterious microbes but also tissue damage[7,8,37,38]. We have called this "secondary immune surveillance" as these CLRs are activated by TLR signaling. We hypothesize that secondary immune surveillance is a way the innate immune system can quickly and rapidly diversify what the immune system is able to detect after an initial or primary immune threat, like gut leakage.

To better understand secondary immune surveillance, we need to determine how CLRs are transcriptionally activated by innate immune signaling. Mincle (Clec4e) is upregulated by LPS (TLR4) signaling, and previous work from our lab found that other CLRs in the NK gene receptor complex are also upregulated by LPS. Additionally, these CLRs, which include Mincle (Clec4e), Dectin-3 (Clec4d), and Dectin-2 (Clec6a), are highly co-regulated in human monocytes. The genes for these three

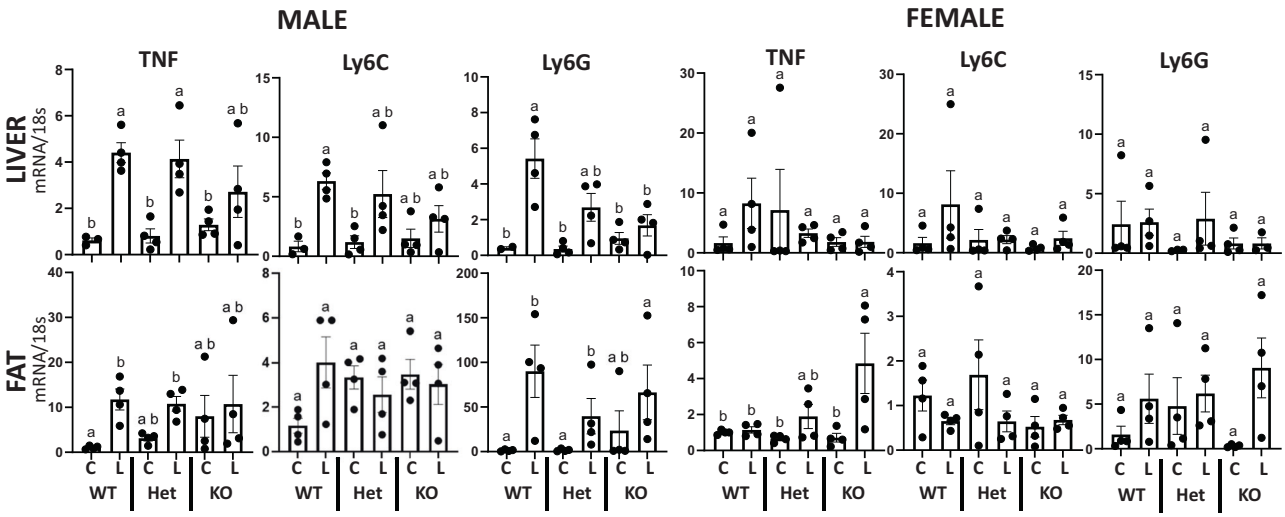

Fig. 5 | qPCR results of RNA isolated from mouse liver and gonadal adipose tissue after LPS injection (0.7 mg/kg). Data are expressed as mean ± SEM. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparisons test ($p < 0.05$); groups not sharing the same letter are significantly different.

receptors are very closely associated in the genome and occupy the same topologically-associated domain (TAD)[20].

The purpose of this study was to broaden our understanding of CLRs and secondary immune surveillance in innate immunity beyond what is already known regarding Mincle. Most studies in the field have focused on Mincle gene regulation, downstream pro-inflammatory signaling, and the role Mincle has on the liver during chronic disease. In this study, we hypothesized that many of these CLRs are regulated in the same way as Mincle, which means their individual and combined impact on innate immunity is very complicated. Additionally, the NK gene receptor complex contains a large number of CLRs, so it is likely that not all of these genes are regulated the same way as Mincle, but they might all contribute to secondary immune surveillance.

First, we found that Mincle, Dectin-3, and Dectin-1 are transcriptionally upregulated by multiple TLR agonists and interferon alpha, meaning these genes are upregulated by both NF-κB and interferon response elements. An early study had already shown that Mincle is upregulated by LPS, TNFα, IL-6, and IFN-γ[39], so here we expand the types of PAMPs capable of inducing expression of Mincle and other CLRs. These results imply that secondary immune surveillance is activated in response to almost all primary innate immune triggers. Second, we found that Mincle agonists also signal through both NF-κB and interferon pathways, suggesting a possible feed forward loop of CLR regulation.

Almost all of the work on CLR transcriptional upregulation has been performed on human monocytes, either peripheral blood mononuclear cells[19] or in this study, THP-1 monocytes. To extend our understanding of this region of the genome, we looked at how mouse monocytes express these genes. Both species have a similar NK gene receptor complex, though many of the genes have expanded in mice, such that the complexity is far greater in the mouse genome. Using publicly available scRNA-seq data from mice after an in vivo LPS challenge, we found extensive CLR upregulation in mouse circulating monocytes and in microglia in the brain. These data suggest that CLR upregulation is an evolutionarily conserved feature of innate immunity and that they are upregulated by monocytes and more differentiated macrophages.

Previous studies found that Mincle is a critical CLR for liver infiltrating immune cells, and that Mincle-KO mice are protected from chronic liver inflammation caused by alcohol. But what is unclear from these studies is what happens to the expression of the other CLRs that are co-regulated with Mincle, like Dectin-3, and what might the upregulation of these other genes mean for systemic inflammation? Using the Mincle-KO, Mincle-Het, and WT mice, we challenged these mice with low-dose LPS as a way to acutely challenge the immune system. At this concentration of LPS, mice do not experience liver damage, but there is inflammation in the liver with increased infiltrating monocytes and neutrophils. The Mincle-KO mice were protected from liver inflammation, as expected. But the Mincle-KO mice did experience far worse adipose tissue inflammation. Immune cells infiltrate tissues experiencing damage, so in the Mincle-KO mice, blocking immune cells from infiltrating the liver probably caused more inflammation in the adipose tissue. This would suggest that while Mincle may contribute significantly to the progression of chronic liver diseases like ALD, it has an important role in helping the liver to response to acute infections, and when this is lost, other tissues are affected. It is important to note that we observed a significant sex difference in terms of responce to LPS. While male mice experienced liver and adipose tissue inflammation after LPS injection, female mice did not. One possible explanation for this is difference in TLR4 expression between males and females, due in part to testosterone inhibiting TLR4 expression[40]. Our results contribute to the growing body of data suggesting sex differences in innate immunity leads to greater risk of infectious disease in males than females, and that this might extend to liver and adipose tissue infections.

Other studies have found that Mincle-KO mice have increased gut leakage and bacterial translocation due to reduced detection of commensal bacteria and thus reduced anti-microbial peptide production[31]. But to complicate matters further, multiple studies have found that Mincle-KO mice or mice given a Mincle inhibitory aptamer are protected from mouse models of colitis[41,42]. Together, these results all suggest an important role Mincle has in maintaining homeostasis in the gut-liver-adipose tissue axis, but during chronic disease, Mincle can worsen inflammation between these three sites.

Finally, Mincle had previously been shown to play a significant role in the progression of ALD in murine models as well as in peripheral immune cells in AH[9,19]. We found that almost all other CLRs in the NK gene receptor complex were up or downregulated in AH. These CLRs are functionally very diverse, with expression on NK cells and diverse myeloid cells like dendritic cells, monocytes, macrophages, and neutrophils. Additionally, as receptors, they are able to detect a wide variety of PAMPs and DAMPs but also other receptors to activate or repress signaling. For example, Klrc1, Klrc2, and Klrg1 are important for cytotoxic T-cell and NK-cell targeting, and previous work from out lab found reduced expression of these genes in peripheral immune cells, suggesting a difference in expression between peripheral NK cells and the liver[43]. Because of this diversity, these genes open up a wide variety of potential targets to reduce inflammation in severe AH.

In conclusion, secondary immune surveillance is broadly upregulated by innate immune cells in response to inflammatory signaling. CLRs play an important role in responding to infectious agents but can also contribute to tissue specific inflammation. These receptors are upregulated in AH livers

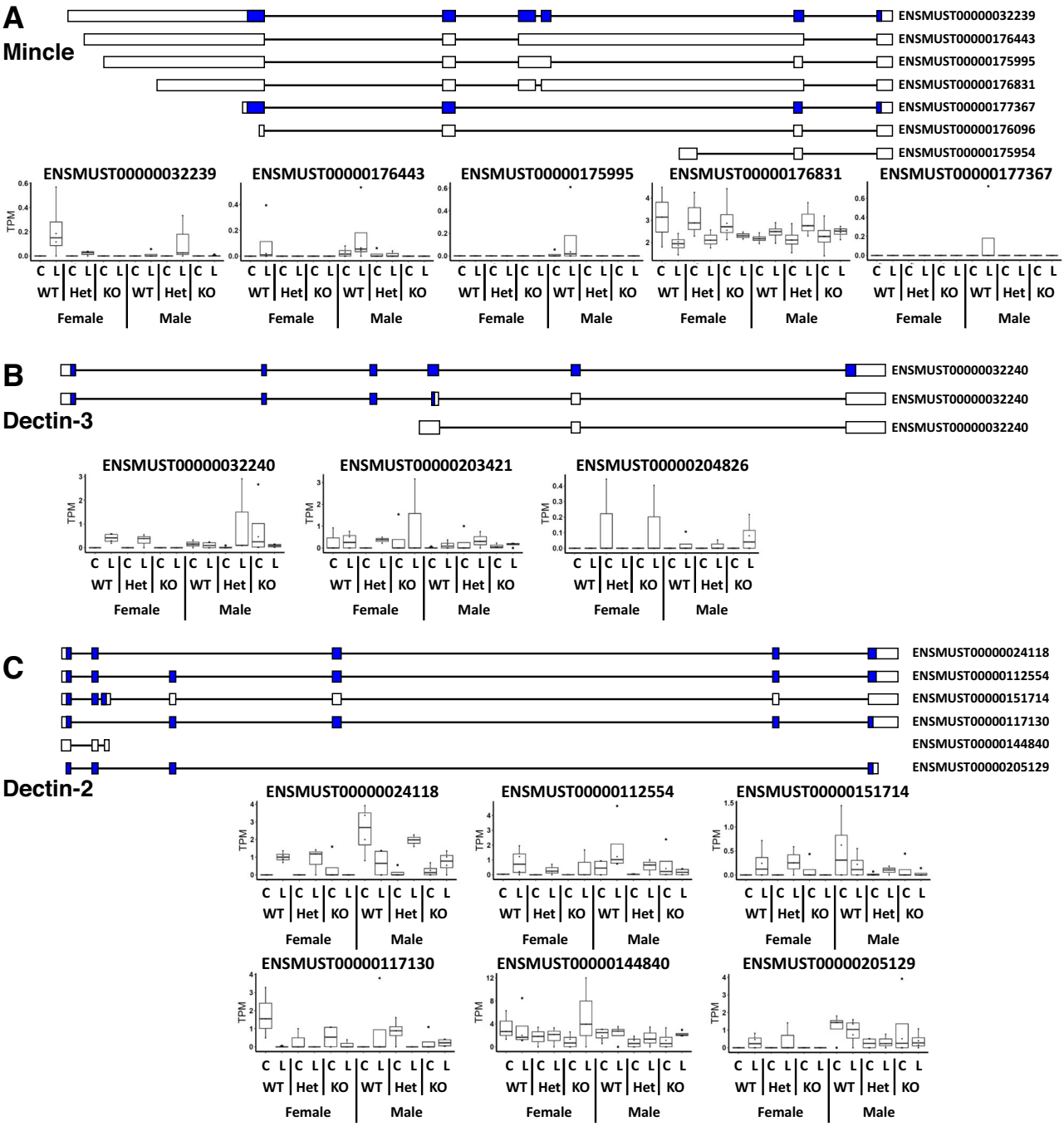

Fig. 6 | Bulk RNA-seq of livers from WT, Mincle-Het, and Mincle-KO with and without low-dose LPS injection. Expression of distinct splice isoforms of CLR genes, including (**A**) Mincle/Clec4e, (**B**) Dectin-3/Clec4d, and (**C**) Dectin-2/Clec4n.Exons in blue indicate coding regions of the mRNA, and mRNAs without blue exons are not thought to be transcribed.

and peripheral immune cells, and understanding how they are regulated and their function in inflammation may lead to novel therapies for this disease.

Materials and methods

THP1-Dual cell culture

THP1-Dual™ (InvivoGen, USA) cell line was cultured in RPMI 1640 medium containing 2 mM L-glutamine, 25 mM HEPES, 10% heat-inactivated fetal bovine serum (30 min at 56 °C), 100 µg/ml Normocin and 100 U/ml Pen-Strep. To maintain selection pressure, 10 µg/mL of Blasticidin and 100 mg/mL of Zeocin antibiotics were added to the growth medium every other passage.

THP1-Dual™ cells were plated in a 96-well plate at a concentration of 5.56×10^5 cells/ml per well ($n = 3$ for all experimental conditions). Cells ($n = 3$) were challenged with different concentrations Pam3CSK4 (InvivoGen, San Diego, CA), R848 (InvivoGen, San Diego, CA), Lipopolysaccharide (LPS) (*Escherichia coli* strain L2654(O26:B6), Sigma-Aldrich, Burlington, MA), and Human IFN-alpha 2 (Peprotech, Cranbury, NJ). After 24 h incubation, cells were pelleted by centrifugation at $300 \times g$ for 5 min, then supernatant was collected. SEAP and Luciferase results were measured from cell supernatant following manufacturer instructions. Cells were also challenged with LPS (100 pg/ml) for 24 h, then TDB (10 µg/ml,

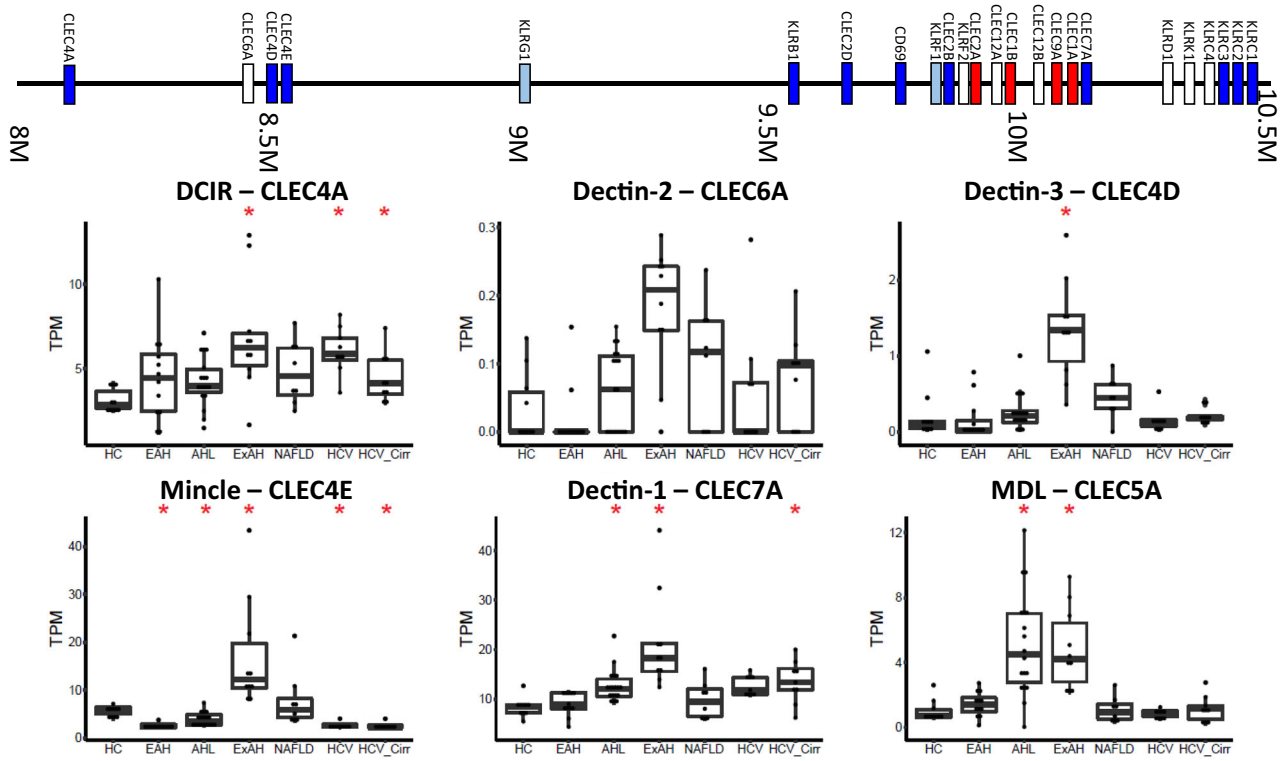

Fig. 7 | Bulk RNA-seq of livers from patients with different chronic diseases. **A** Schematic of genes in the NK-gene receptor complex. Blue = genes upregulated in severe AH, Red = genes downregulated in severe AH. **B** Boxplots of specific CLR genes. HC-Healthy Control, EAH-Early AH, AHL-Severe AH with Liver Failure, ExAH-Explant tissue from severe AH, NAFLD-Nonalcoholic Fatty Liver Disease, HCV-Hepatitis C Virus, HCV-Cirr-HCV with Cirrhosis. MDL/Clec5a is a CLR that is not in the NK-gene receptor complex. * Indicates $q < 0.05$ compared to HC.

Invivogen, San Diego, CA) for 24 h, then supernatant was collected and RNA extracted from cells.

Mice

All animals received humane care. WT C57BL/6 J mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mincle-KO mice obtained from Osaka University, who generated the mice[7,9,44]. All procedures using animals were approved by the UConn Health Institutional Animal Care and Use Committee.

Mincle-Het mice were crossed to generate WT, Mincle-Het, and Mincle-KO mice, as determined by genotyping. At 5–6 months of age, mice were weighed, then injected by intraperitoneal (IP) injection with 0.7 g/kg LPS or saline[29]. 24 h after LPS injection, mice were euthanized under anesthesia via IP injection of a combination of xylazine (10 mg/kg body weight), ketamine (80 mg/kg body weight), and acepromazine cocktail. Blood, adipose tissue, and liver samples were harvested for further analysis. Portions of liver and epididymal adipose tissue were flash frozen in liquid nitrogen and stored at −80 °C until RNA isolation or fixed in 10% formalin for histology. Blood was transferred to EDTA-containing tubes for the isolation of plasma. Plasma was then stored at −80 °C.

Biochemical assays

Plasma samples were assayed for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using a commercially available enzymatic assay kit (Sekisui Diagnostics, Lexington, MA), following the manufacturer's instructions.

Histopathology

Formalin-fixed liver tissues were paraffin-embedded, sectioned and stained with hematoxylin and eosin for histological analysis. Tissues were coded at time of collection to assure an unbiased analysis; at least 3 images were acquired per tissue section.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was extracted by using the Direct-zol mini kit per the manufacturer's instructions (Zymo Research, Irvine, CA). For mRNA measurement, RNA was reverse-transcribed using the Superscript Vilo kit (Thermo Fischer, Waltham, MA) and measured with PowerSYBR qRT-PCR kits (Applied Biosystems, Foster City, CA) on a QuantStudio5 analyzer (Applied Biosystems, Foster City, CA). Relative messenger RNA (mRNA) expression was determined using gene-specific primers. Analyses were performed using the ΔΔCt method (average Ct of gene of interest–average Ct of 18S).

Bulk RNA sequencing of Mouse Liver

We extracted RNA from flash frozen liver tissue using the Zymo Direct-zol kit with an in-column DNAse step. 1 ug of RNA was used to prepare Illumina stranded RNA-seq libraries using the SMART-Seq® Total RNA High Input (RiboGone™Mammalian) kit (Takara Bio, San Jose, CA). Libraries were multiplexed and sequenced on a and sequenced them on an Illumina NovaSeq to generate more than 2 billion paired-end reads of 100 bp with a median of 18 million reads per sample.

Read alignment

Fastq files were aligned to the mouse genome (GRCm39, release 113) using Kallisto version 0.46.1 with 100 bootstraps calculated[45]. Data were then merged with experimental data and analyzed with Sleuth in transcript_mode and in gene_mode with aggregation_column set to Ensemble Gene ID; in addition, extra_bootstrap_summary and

read_bootstrap_tpm were set to true[46]. Differential expression was measured with Sleuth using a cutoff of $q < 0.05$.

Analysis of single cell RNA-sequencing Data from Mouse Monocytes

scRNA-seq data were obtained, already aligned, from the primary publication [GEO: GSE160450][26]. All gene expression and clustering analyses were performed using Seurat[47]. All samples were combined using the PrepSCTIntegration and FindIntegrationAnchors functions to find common anchor genes in all samples for all cell types, and then integrated using the IntegrateData function, with all normalizations using the SCT transformed data. Clustering was performed using RunPCA and RunUMAP, and clusters were identified using FindNeighbors and FindClusters.

Analysis of Bulk RNA-sequencing Data from Mouse Microglia

Raw sequencing data (fastq files) were obtained from the original publication [GEO: GSE75246][27]. Fastq files were aligned to the mouse genome (GRCm39, release 113) using Kallisto version 0.46.1 with 100 bootstraps calculated[45]. Data were then merged with experimental data and analyzed with Sleuth in gene_mode with aggregation_column set to Ensemble Gene ID; in addition, extra_bootstrap_summary and read_bootstrap_tpm were set to true[46]. Differential expression was measured with Sleuth using a cutoff of $q < 0.05$.

Analysis of Bulk RNA-sequencing data from liver-disease patients

Data was analyzed as described previously[19,48]. In summary, all raw fastq files were downloaded from SRA [PRJNA531223] and dbGAP [phs001807.v1.p1][30]. Fastq files were aligned to the human genome (GRCh38, indices downloaded from https://github.com/pachterlab/kallisto-transcriptome-indices/releases/download/ensembl-96/homo_sapiens.tar.gz) using Kallisto version 0.44.0 with 100 bootstraps calculated[45]. Data were then merged with clinical data and analyzed with Sleuth in gene_mode with aggregation_column set to Ensemble Gene ID; in addition, extra_bootstrap_summary and read_bootstrap_tpm were set to true[46]. Differential expression was measured with Sleuth using a cutoff of $q < 0.05$.

Statistical analysis for qPCR

Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparisons test ($p < 0.05$). Statistical difference between groups was determined at $p < 0.05$ and indicated by asterisks.

Data availability

The bulk RNA-seq data from mouse livers generated in this study can be found at National Center for Biotechnology Information Gene Expression Omnibus under accession number PRJNA1436629. All other data used in this study is publicly available: Single-cell RNA-seq from mouse monocytes [GSE160450][26], Bulk RNA-seq from mouse microglia [GSE75246][27], Bulk RNA-seq from human liver [PRJNA531223] and dbGAP [phs001807.v1.p1][30].

Code availability

Bulk RNA sequencing data for this study can be found at NCBI GEO under accession number PRJNA1436629. All scripts used for analyses can be found at author's github (github/atomadam2).

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Author contributions

F.P., E.B., Y.Z., C.V., and A.K. contributed to the conception and design of the study. F.P., E.B., Y.Z., and C.V. designed and performed all experiments. A.K. analyzed the scRNA-seq and bulk RNA-seq data. A.K. wrote the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

Competing interests

The authors declare no competing interests.

Additional information

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